AD_____

GRANT NUMBER DAMD17-98-1-8223

TITLE: Developing Strategies to Block Beta-Catenin Action in Signaling and Cell Adhesion during Carcinogenesis

PRINCIPAL INVESTIGATOR: Mark A. Peifer, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina
Chapel Hill, North Carolina 27599-1350

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE
Developing Strategies to Block Beta-Catenin Action in Signaling and Cell Adhesion during Carcinogenesis

5. FUNDING NUMBERS
DAMD17-98-1-8223

6. AUTHOR(S)
Mark A. Peifer, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina
Chapel Hill, North Carolina 27599-1350

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

To understand abnormal cell behavior in cancer, we must understand normal cell behavior. We focus on *Drosophila* Armadillo (Arm) and its human homolog ß-catenin. Both are key players in two processes: 1) They are components of cell-cell adhesive junctions, and 2) they act in transduction of Wingless (Wg)/Wnt cell-cell signals. Mutations in ß-catenin or its regulators are early steps in certain cancers. Our working hypotheses are: 1) Several protein partners compete to bind to the same site on Arm, and 2) The Arm:dTCF complex activates Wg-responsive genes; dTCF can represses the same genes. Our Aims are to understand : 1) how different partners interact with and compete with one another for binding Arm, and 2) how the Arm partner dTCF positively and negatively regulates Wg responsive genes. In the past year we made significant progress on both goals. With regard to Aim 1, we further defined the Arm binding sites on dTCF, DE-cadherin, dAPC, and dAPC2 and analyzed the effect of point mutations within the DE-cadherin site on binding. We also initiated analysis of binding in mammalian cells with collaborators at the Weizmann Institute. With regard to Aim 2, we found that dTCF is a repressor of Wingless-responsive genes in the absence of Arm, and that Groucho acts as a co-repressor. These data were published in Nature. We also found that Arm's C-terminus has several roles in addition to its role as a transcriptional activation domain.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer Beta-catenin, Armadillo, Wingless/Wnt, APC	16
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N.A. Where copyrighted material is quoted, permission has been obtained to use such material.

N.A. Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ MP Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ MP For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

M Ayala 7/27/99

PI - Signature Date

(4) Table of Contents

(5) Introduction:

To understand abnormal cell behavior in cancer, we must first understand normal cell behavior. We focus on *Drosophila* Armadillo (Arm); Arm and its human homolog ß-catenin are critical for normal embryonic development (reviewed in Peifer, 1997). Both are key players in two separable biological processes: 1) They are components of cell-cell adhesive junctions, and 2) they act in transduction of Wingless/Wnt (Wg/Wnt) family cell-cell signals (Peifer, 1995). Mutations in ß-catenin or its regulators are early steps in colon cancer and melanoma. We use the fruit fly as our model, combining classical and molecular genetics with cell biology and biochemistry. We take advantage of the speed and ease of the fly system and of its synergy with vertebrate cell biology. As one avenue to reveal Arm's roles in adherens junctions and transduction of Wg signal, we are identifying and examining the function of proteins with which Arm physically and/or functionally interacts. Our goal is to precisely define Arm/ß-catenin's dual roles, ultimately allowing the design of drugs inhibiting oncogenic ß-catenin. Our working hypotheses are: 1) Several protein partners compete to bind to the same site on Arm; the affinity of Arm for different partners is adjusted via phosphorylation of these partners, and 2) The Arm:dTCF complex activates Wg-responsive genes; dTCF represses the same genes in the absence of Arm. We will integrate approaches at all levels from combinatorial chemistry to studying gene function in intact animals, using fruit flies to carry out a functional genomics approach to understanding Arm function, and then transferring this knowledge directly to the mammalian system. Our first Aim is to understand how different partners interact with and compete with one another for binding Arm, and how phosphorylation regulates this. Our second Aim focuses on how the Arm partner dTCF positively and negatively regulates Wg responsive genes.

Specific Aim 1. Identify the sequence determinants mediating the binding of Armadillo/ß-catenin's protein partners to Armadillo/ß-catenin.

Specific Aim 2. Explore the mechanism of action of dTCF, a Wingless/Wnt effector.

We have made significant progress on both of these Specific Aims, which we have outlined below.

(6) Body:

Our statement of work for the first aim for the first year stated:

Year 1

1. Minimize interacting regions of all three partners and begin mutagenesis.
2. Carry out two-hybrid screen for random peptides that interact with Arm.
3. Mutagenize & test in two-hybrid system potential phosphorylation sites.

At the time of submission, we knew that dAPC, DE-cadherin, and dTCF all can bind to ~260 amino acids comprising Arm's Arm repeats 3-8 (Pai et al., 1996; van de Wetering et al., 1997; unpublished data). We had begun to examine the regions of each of these partner proteins which bind to Arm. At that time we knew that a 40 amino acid region of the DE-cadherin cytoplasmic tail (Pai, et al., 1996; Fig. 1), a 70 amino acid region at the N-terminus of dTCF (van de Wetering et al., 1997), and a 120 amino acid region of dAPC were sufficient for binding to Arm (using the yeast two-hybrid system as an assay).. We have substantially extended these findings, in pursuit of task 1 in the statement of work. We now have found that a 30 amino acid region of the cadherin tail can mediate binding (Fig. 1), and we are currently making smaller constructs to test in the binding assay. We have also found that a 42 amino acid region of dTCF can mediate interaction with Arm (Fig. 2), and are also making smaller pieces to test for binding. Finally, we have shown that 31-34 amino acid pieces of dAPC, carrying individual 15 or 20 amino acids repeats (identified as the ß-catenin binding sites in human APC), can also bind Arm, and that they each bind to the core Arm repeat region (repeats 3-8). In studies funded by the NIH and the HFSP, we have identified a second fly APC protein, which we call dAPC2 (van Es et al., 1999). We have extended our studies to this protein, and have found that 30-31 amino acid pieces carrying individual 15 and 20 amino acid repeats of dAPC2 are also sufficient for Arm binding.

We have further extended these observations by beginning to examine the sequence requirements for Arm binding, beginning our examination by focusing on the DE-cadherin target. We based these experiments on a slight but intriguing sequence similarity between Arm's partners (Fig. 1). In particular, the motif SLSSL is conserved in APC and cadherin. This is of special interest because vertebrate E-cadherin and APC are phosphorylated in this region, most likely on these serines. In APC, phosphorylation of these serines by GSK-3 enhances ß-catenin binding (Rubinfeld et al., 1996). In E-cadherin, serines in the region are phosphorylated by an unknown kinase; mutation of the serines to alanine blocks ß-catenin binding (Stappert and Kemler, 1994). We thus made an extensive series of site-directed mutations of conserved residues (including serines) within the minimal Arm binding region, including a small deletion and clustered point mutations, as outlined in parts one and three of the statement of work. To our surprise, most of these mutations do not block binding to Arm when tested in the context of the full length cadherin tail (Fig. 3). This suggests that multiple points of contact may underlie binding and that changes in individual contact sites may not be sufficient to block the interaction.

To supplement this work using the yeast two-hybrid system, we have initiated a collaboration with Avri Ben'Zeev and Benny Geiger of the Weizmann Institute in Israel. They have begun to test our wild-type and mutant DE-cadherin constructs, as well as our minimal dAPC and dTCF constructs, for their ability to bind to ß-catenin in mammalian cells, when expressed as GFP-fusion proteins. They are examining the localization of these fusion proteins, as well as their ability to block destruction of endogenous ß-catenin or to block activation by the ß-catenin LEF complex. We have not yet begun the work outlined in part two of the statement of work, as the work on the other sections has gone well and we have chosen to focus our effort on these sections.

We have also initiated a new aspect of the project. In order for Armadillo or ß-catenin to interact with TCF family members and activate transcription, they must enter the nucleus. The mechanism by which this occurs is of great interest. Both proteins lack classical NLS sequences and thus must be imported by a novel mechanism. Work from the Gumbiner lab has shown that ß-catenin can import itself without assistance from the standard importin-based nuclear import machinery (Fagotto et al., 1998). Recently, a system has been developed which allows one to assess sequences required for nuclear import in yeast, via a variant of the yeast two-hybrid system (Ueki et al., 1998). The workers characterizing this system found that the C-terminal half of ß-catenin could direct nuclear import of a heterologous protein. We have obtained these vectors and have begun mapping the sequences necessary for Armadillo nuclear import-- when we have completed this analysis, we can mutate these sequences and test the resulting mutant proteins in flies.

Armadillo:dTCF, a bipartite transcription factor

For this aim, our statement of Work for the first year listed the following goals

1. Construct, introduce into flies and begin to test effects of *arm* mutants with C-termini replaced with known activation and repression domains.
2. Examine genetic interactions between *gro*, *wg* ,*arm* and *dTCF* mutations.

We have made significant progress in our work on the role of dTCF as a repressor of Wg-responsive genes (Part 2 of the Statement of Work above), in collaboration with Amy Bejsovec of Northwestern and Hans Clevers in Utrecht. In addition to the preliminary data described in our original application, we found that *dTCF* mutations suppress the segment polarity phenotype of a second *arm* allele, that the constitutive repressor form of dTCF (dTCF_N) represses expression of the Wg-responsive gene En, and finally that expression of excess dTCF could enhance the phenotype of a weak allele of *wg,* and also further repress expression of En in this background. Together with our previous data, these data strongly support a model in which dTCF, when not bound to Arm, acts as a dose-sensitive repressor of Wg-responsive genes.

We also explored the idea that Groucho may act as a repressor of Wg-responsive genes (part 2 of the Statement of Work above). We have found that *Drosophila* Groucho can bind to dTCF when they are co-expressed in mammalian cells, and that it can antagonize gene activation by the dTCF-Arm complex. Second, we have found that reduction in Groucho dose suppresses the segment polarity phenotype of *wg* and *arm* mutations, and partially relieves repression of the Wg-responsive gene En. Finally, we found that reduction in the dose of Gro reduces the phenotypic effect of the constitutive repressor form of dTCF, dTCF_N. These data were published in a paper in Nature, with partial support from our Army grant (Cavallo et al., 1998; a reprint is included in the Appendix).

We also have explored in more detail the role of Arm's C-terminus in Wg signaling (Part 1 of the Statement of Work above). *Drosophila melanogaster* Armadillo and its vertebrate homolog ß-catenin play multiple roles during development. Both are components of cell-cell adherens junctions and both transduce Wingless/Wnt intercellular signals. The current model for Wingless signaling proposes that Armadillo binds the DNA-binding protein dTCF, forming a bipartite transcription factor which activates Wingless-responsive genes. In this model, Armadillo's C-terminal domain was proposed to serve an essential role as a transcriptional activation domain. However, in Xenopus overexpression of C-terminally truncated ß-catenin activates Wnt signaling (e.g., Funayama et al., 1995), suggesting that the C-terminal domain might not be essential. We re-examined the function of Armadillo's C-terminus in Wingless signaling. We found that C-terminally truncated mutant Armadillo has a deficit in Wg signaling activity, even when corrected for reduced protein levels. However, we also found that Armadillo proteins lacking all or part of the C-terminus retain some signaling ability if overexpressed, and that mutants lacking different portions of the C-terminal domain differ in their level of signaling ability. Finally, we found that the C-terminus plays a role in Armadillo protein stability in response to Wingless signal, and that the C-terminal domain can physically interact with the Arm repeat region. These data suggest that the C-terminal domain plays a complex role in Wingless signaling, and that Armadillo recruits the transcriptional machinery via multiple contact sites, which act in an additive fashion. These data are now in press in Genetics, with partial support from our Army grant (reprints are not yet available but will included with next years update).

(7) Key research accomplishments.

a) 30-35 amino acids of dTCF, DE-cadherin and dAPC1 and dAPC2 are each sufficient for Armadillo binding in the yeast two-hybrid system.

b) Point mutations in conserved sequence motifs in DE-cadherin do not block Armadillo binding.
c) dTCF represses as well as activates Wingless responsive genes.
d) Groucho acts as a co-repressor for dTCF in repression of Wg-responsive genes.
e) Armadillo's C-terminus plays multiple roles in Armadillo function.

(8) Reportable outcomes.

Publications supported in part by this grant:

Cox, R.T., Pai,L.-M., Kirkpatrick, C., Stein,J., and Peifer, M. (1999). Roles of the C-terminus of Armadillo in Wingless signaling in *Drosophila* . Genetics, in press (reprint not yet available-- it will be included in next years report).

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). *Drosophila* TCF and Groucho interact to repress Wingless signaling activity. Nature 395, 604-608. (copy included in appendix)

Presentations by Mark Peifer discussing this work.

"Cell adhesion, signal transduction and cancer: the Armadillo Connection.", Inaugural Symposium for the Developmental Genetics Programme and British Biochemical Society Annual Meeting, Krebs Institute,Sheffield, England, United Kingdom, July 1998

"Cell adhesion, signal transduction and cancer: the Armadillo Connection.", Annual Meeting, British Society of Cell Biology, Oxford, England, United Kingdom, September 1998

"Cell adhesion and signal transduction: the Armadillo Connection." ERDA Program, NIEHS, RTP NC September, 1998

"Cell adhesion and signal transduction: the Armadillo Connection." Department of Pharmocology, University of North Carolina at Chapel Hill, Chapel Hill NC October, 1998

"Cell adhesion and signal transduction: the Armadillo Connection." Department of Molecular Biosciences, University of Kansas, Lawrence KS February, 1999

"Cell adhesion and signal transduction: the Armadillo Connection." Hubrecht Laboratory of Developmental Biology, Dutch National Science Foundation, Utrecht, the Netherlands, March, 1999

"Cell adhesion and signal transduction: the Armadillo Connection." Dutch National Cancer Institute, Amsterdam, the Netherlands, March, 1999

"Cell adhesion and signal transduction: the Armadillo Connection." Department of Developmental and Cell Biology, University of California, Irvine CA, May 1999.

"Cell adhesion, signal transduction and cancer: the Armadillo Connection." Cell Contact and Adhesion Gordon Conference, Andover NH June 1999.

(9) Conclusions.

We have made significant progress on each of the specific aims. We have focused in on the key regions of dTCF, DE-cadherin, dAPC and dAPC2 that mediate Arm binding, and have initiated work in mammalian cells, in collaboration with our colleagues at the Weizmann Institute. We anticipate completing the initial phase of this work within the next year. We have also begun to examine the sequence requirements for nuclear import, using a simple, yeast based assay. These data should provide a basis for understanding the interaction between the oncogene ß-catenin and its mammalian partners in both normal development and physiology, and during oncogenesis.

Our work on dTCF and Groucho resulted in a publication in Nature. This work has stimulated parallel work by others on vertebrate Groucho homologs, which have been revealed to play a role in TCF-mediated repression of Wnt responsive genes. Understanding the mechanism by which such genes are repressed will provide insight into the normal and abnormal regulation of the genes responsible for oncogenesis in tumors resulting from activation of the Wnt pathway.

(10) References.

Cavallo, R. A., R. T. Cox, M. M. Moline, J. Roose, G. A. Polevoy, H. Clevers, M. Peifer and A. Bejsovec. 1998. *Drosophila* TCF and Groucho interact to repress Wingless signaling activity. *Nature* 395: 604-608.

Fagotto, F., U. Glück and B. M. Gumbiner. 1998. NLS- and importin/karyopherin-independent nuclear import of ß-catenin. *Curr. Biol.* 8: 181-190.

Funayama, N., F. Fagatto, P. McCrea and B. M. Gumbiner. 1995. Embryonic axis induction by the Armadillo repeat domain of ß-catenin: evidence for intracellular signaling. *J. Cell Biol.* 128: 959-968.

Pai, L.-M., C. Kirkpatrick, J. Blanton, H. Oda, M. Takeichi and M. Peifer. 1996. *Drosophila* a-catenin and E-cadherin bind to distinct regions of *Drosophila* Armadillo. *J. Biol. Chem.* 271: 32411-32420.

Peifer, M. 1995. Cell adhesion and signal transduction: the Armadillo connection. *Trends Cell Biol.* 5: 224-229.

Peifer, M. 1997. ß-catenin as oncogene: the smoking gun. *Science* 275: 1752-1753.

Rubinfeld, B., I. Albert, E. Porfiri, C. Fiol, S. Munemitsu and P. Polakis. 1996. Binding of GSK-ß to the APC/ß-catenin complex and regulation of complex assembly. *Science* 272: 1023-1026.

Stappert, J. and R. Kemler. 1994. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhesion Commun.* 2: 319-327.

Ueki, N., T. Oda, M. Kondo, K. Yano, T. Noguchi and M. Muramatsu. 1998. Selection system for genes encoding nuclear-targeted proteins. *Nat. Biotechno.* 16: 1338-1342.

van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin and H. Clevers. 1997. Armadillo co-activates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. *Cell* 88: 789-799.

van Es, J. H., C. Kirkpatrick, M. van de Wetering, M. Molenaar, A. Miles, J. Kuipers, O. Destrée, M. Peifer and H. Clevers. 1999. Identification of APC2, a homologue of the adenomatous polyposis coli tumour suppressor. *Curr. Biol.* 9: 105-108.

Appendix I-- Figures

Figure 1. Conserved regions of the Arm-binding region of cadherin-target for mutagenesis
Minimal region for binding to Armadillo is overlined

```
DE-cad      KKENCDRDVGATTVDDVRHYAYEGDGNSDGSLSSLASCTDDGDLNFDYLS.NFGPRFRKLADMYGEEPSDT
mOB-cad     RIQEADNDPTAPPYDSIQIYGYEGRGSVAGSLSSLESATT DSDLDYDYLQNWGPRFKKLADLYGSKDTFD
mE-cad      NLKAADSDPTAPPYDSLLVFDYEGSGSEAASLSSLNSSESDQDQDYDYLNE.WGNRFKKLADMYGGGE..D
```

Conserved potential phosphorylation sites * ** *
Minimal region for binding to ß-catenin is underlined (Stappert and Kemler, 1994)

The Armadillo binding regions of known partners share a sequence motif

```
Xenopus TCF-3 MPQLNSGGGD--DELGANDELIRFKDEGE-QEEKSPGEGSAEGDLADVKSSLVN--ESE
dTCF          MPHTHSRHGSSGDDLCSTDEVKIFKDEGDREDEKI----SSENLLVEEKSSLIDLTESE
mouse OB-CAD      RIQEADNDPTAPPYDSIQIYGYEGRGSVA----------GSLSSLNS-SESD
dECAD             KKENCDRDVGATTVDDVRHYAYEGDGNSD----------GSLSSLASCTDDG
APC concensus                                  FXVEXTPXCFSRXSSLSSL S
Phosphorylation sites in APC and E-cadherin                 * **  *
```

The mimimal binding site on DEC for Arm is 30 amino acids (DEC 13)

DE-cadherin constructs

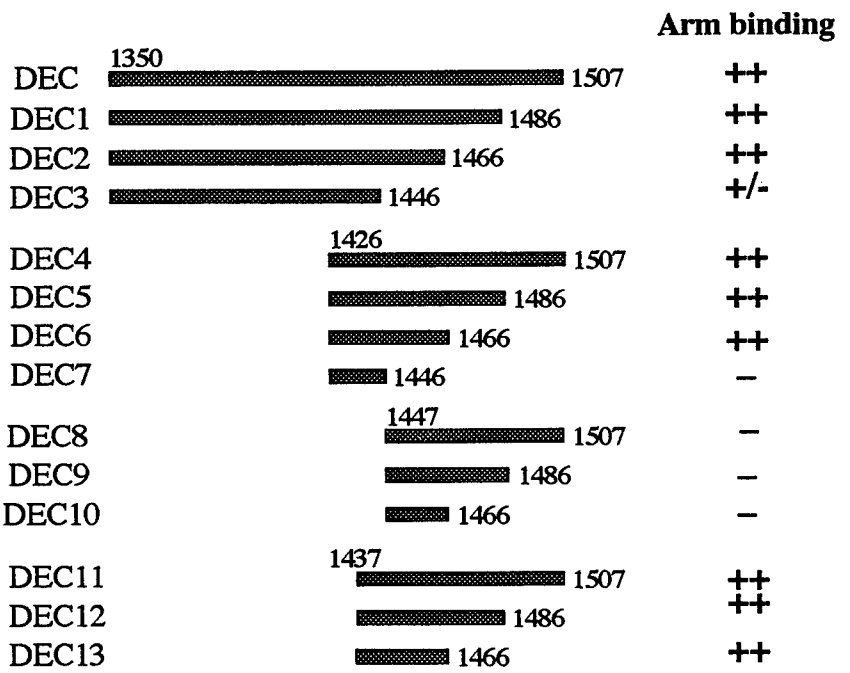


Fig. 2. Minimal region of dTCF that can mediate binding of dTCF to Arm. Numbers indicate amino acid residues of dTCF.

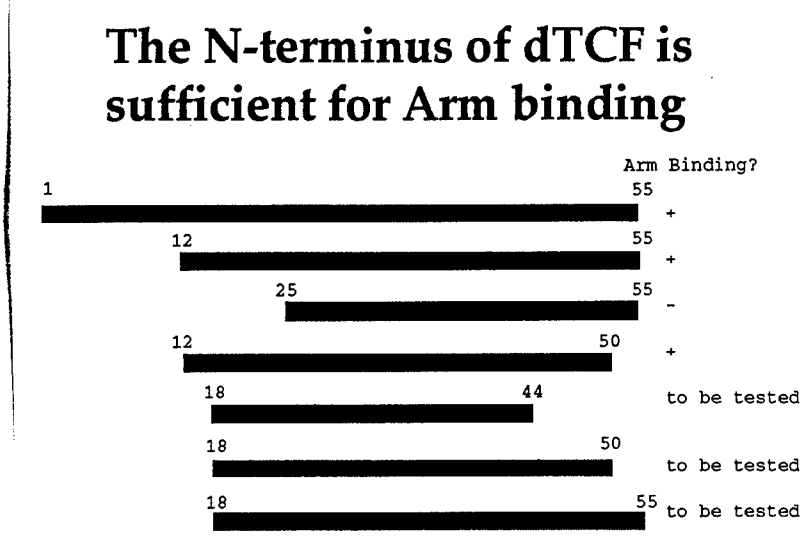
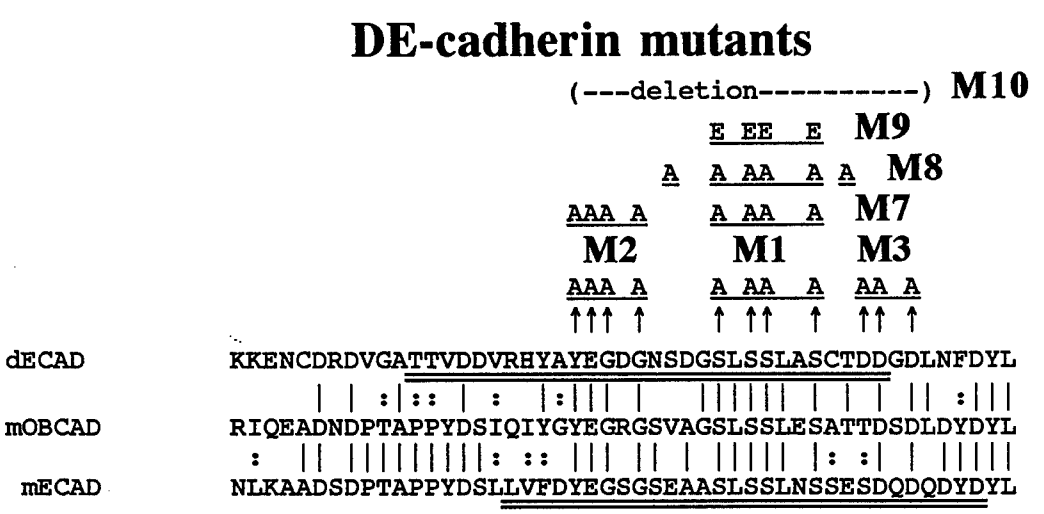
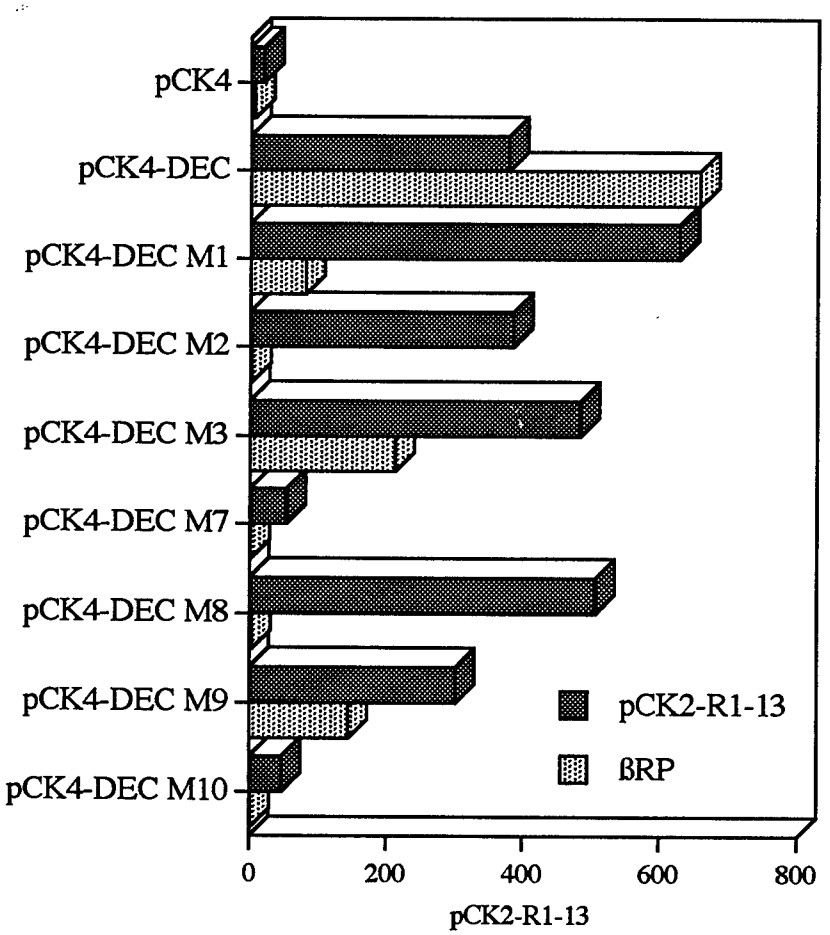


Fig. 3 Defining the Arm binding site on DE-cadherin. A. Diagram of mutations induced in DE-cadherin. All were tested in the context of the full length cytoplasmic tail of DE-cadherin. B. Results of ß-galactosidase assays, in units. pCK4, the empty vector, is the negative control, and pCK4-DEC, the full length DE-cadherin tail, is the positive control.





Drosophila Tcf and Groucho interact to repress Wingless signalling activity

Robert A. Cavallo*†, Rachel T. Cox*†, Melissa M. Moline†‡, Jeroen Roose§, Gordon A. Polevoy*, Hans Clevers§, Mark Peifer* & Amy Bejsovec‡

* *Department of Biology and Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599, USA*
‡ *Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208, USA*
§ *Department of Immunology, University Hospital, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*
† *These authors contributed equally to this work*

Wingless/Wnt signalling directs cell-fate choices during embryonic development[1,2]. Inappropriate reactivation of the pathway causes cancer[3–5]. In *Drosophila*, signal transduction from Wingless stabilizes cytosolic Armadillo[1], which then forms a bipartite transcription factor with the HMG-box protein *Drosophila* Tcf (dTcf) and activates expression of Wingless-responsive genes[6–8]. Here we report that in the absence of Armadillo, dTcf acts as a transcriptional repressor of Wingless-responsive genes, and we show that Groucho acts as a corepressor in this process. Reduction of dTcf activity partially suppresses *wingless* and *armadillo* mutant phenotypes, leading to derepression of Wingless-responsive genes. Furthermore, overexpression of wild-type dTcf enhances the phenotype of a weak *wingless* allele. Finally, mutations in the *Drosophila groucho* gene also suppress *wingless* and *armadillo* mutant phenotypes as Groucho physically interacts with dTcf and is required for its full repressor activity.

Mutations of Tcf-binding sites in the promoters of *Drosophila*

Table 1 Genetic interactions between *arm*, *dTcf* and *gro*

Genetic cross	Per cent in weakest classes
arm^{XP33}/*FM7* × +/y	1 (n = 402)
arm^{XP33}/*FM7* × *Df(4)M62f*/+	21 (n = 559)
arm^{XP33}/*FM7* × *Df(4)M63a*/+	0 (n = 691)
arm^{XP33}/*FM7* × ci^{D}/+	13 (n = 318)
arm^{XP33}/*FM7* × ci^{cell}/+	2 (n = 353)
arm^{XP33}/*FM7* × $dTCF^{1}$/+	15 (n = 350)
arm^{XP33}/*FM7* × $dTCF^{2}$/+	27 (n = 299)
arm^{XP33}/*FM7* × $dTCF^{3}$/+	23 (n = 595)
arm^{XP33}/*FM7* × gro^{BX22}/+	0 (n = 159)
arm^{XP33}/*FM7* × gro^{E48}/+	1 (n = 151)
arm^{XP33}/+; gro^{BX22}/+ × gro^{BX22}/+	8 (n = 121)
arm^{XP33}/+; gro^{E48}/+ × gro^{E48}/+	12 (n = 378)
arm^{XP33}/+; gro^{BX22}/+ × arm^{XP33}/Y^{arm+}	10 (n = 132)
arm^{YD35}/*FM7* × +/y	14 (n = 171)
arm^{YD35}/*FM7* × $dTCF^{2}$/+	46 (n = 321)
arm^{YD35}/*FM7* × $dTCF^{3}$/+	42 (n = 353)
arm^{YD35}/*FM7* × gro^{BX22}/+	10 (n = 169)
arm^{YD35}/*FM7* × gro^{E48}/+	17 (n = 196)
arm^{YD35}/+; gro^{BX22}/+ × gro^{BX22}/+	46 (n = 183)
arm^{YD35}/+; gro^{E48}/+ × gro^{E48}/+	35 (n = 169)
arm^{XM19}/*FM7* × +/y	3 (n = 214)
arm^{XM19}/*FM7* × $dTCF^{2}$/+	46 (n = 291)
arm^{XM19}/*FM7* × $dTCF^{3}$/+	43 (n = 305)
wg^{CX4}/*CyO* × UAS-dTCF/+; *Df(2)DE*/+	99 (n = 216)
wg^{CX4}E22C/++ × UAS-dTCF/+; *Df(2)DE*/+	47 (n = 308)
wg^{CX4}E22C/++; gro^{BX22}/+ male × UAS-dTCF/+; *Df(2)DE*/+	49 (n = 415)
wg^{CX4}E22C/++; gro^{BX22}/+ female × UAS-dTCF/+; *Df(2)DE*/+	94 (n = 223)
E22C-GAL4/+ × UAS-dTCF-ΔN (*line 5*)	3 (n = 259)
gro^{E48}/+; E22C-GAL4/+ × dTCF-ΔN (*line 5*)	97 (n = 206)
E22C-GAL4/+ × dTCF-ΔN (*line 1*)	3 (n = 216)
gro^{E48}/+; E22C-GAL4/+ × dTCF-ΔN (*line 1*)	93 (n = 231)

Cuticles were scored using the criteria in ref. 15. In each case, we calculated the percentage of embryos in the two least severe phenotypic categories.

Ultrabithorax (*Ubx*)[7] or *Xenopus siamois*[9] reduce the level of gene expression in the normal expression domain of the animal, as expected for perturbation of a transcriptional activator. Surprisingly, however, these mutations also result in ectopic gene expression outside the normal domain. This led to the proposal that Tcf proteins may act as repressors[7,9]. We have tested this hypothesis and found that dTcf can function as either an activator or a repressor of Wingless (Wg)-responsive genes depending on the state of the Wg signalling pathway and thus the availability of Armadillo (Arm), dTcf's coactivator.

Reducing the level of zygotic dTcf, by making embryos heterozygous for a null *dTcf* mutation[8], suppresses the segment polarity phenotype that is a consequence of a *wg* null allele (Fig. 1a, b), consistent with a repressive function for dTcf. Indeed, the previously described *dTcf* null mutations[8] were isolated as dominant suppressors of wg^{IL114} (A.B., unpublished observations). Although the function of maternal dTcf has not been determined, we saw no difference in suppression of *wg* mutations when the mutant *dTcf* allele was derived from the mother or the father. This indicates that little, if any, maternal dTcf participates in the repressive effect. Complete loss of zygotic dTcf suppresses the *wg* cuticle pattern defect even more substantially (Fig. 1c), *wg; dTcf* double mutant embryos are larger and exhibit a greater variety of cell types. The double mutant phenotype closely resembles the *dTcf* zygotic null phenotype (Fig. 1d), supporting the idea that the *dTcf* single mutant lacks both repressor and activator function and therefore should be insensitive to the removal of Wg, the upstream activator.

To determine whether, in the absence of Wg activity, dTcf normally represses Wg target genes, we studied the Wg-responsive gene *engrailed* (*en*), which is expressed in epidermal cells just posterior to the *wg*-expressing row[10–12] and is dependent on Wg activity for maintenance of expression[13,14]. *wg* null mutants completely lose epidermal *en* expression before stage 10 (Fig. 1e), but in homozygous *wg* embryos that are heterozygous for *dTcf*, some cells maintain *en* expression (Fig. 1f). Homozygous *wg; dTcf* mutants maintain more *en* expression than heterozygous mutants (Fig. 1g), similar to that observed in *dTcf* zygotic null mutants[8]. This corroborates a repressive role for dTcf in cells in which the Wg signalling pathway is not active. Thus the severe phenotype of a *wg* null mutant reflects the loss of activation by Arm–dTcf while repression by dTcf remains intact. In contrast, both activation and repression are affected in a *dTcf* zygotic null embryo, resulting in less severe disruption of the cuticle pattern and Wg-responsive gene expression.

We further established that a reduction in Arm levels causes dTcf to act as a repressor. A dose-sensitive screen for suppressors of arm^{XP33} (R.T.C. and M.P., unpublished observations) revealed interactions of arm^{XP33} with *dTcf* that were similar to those between *wg* and *dTcf*. Embryos zygotically mutant for arm^{XP33} show a strong polarity phenotype[15], characterized by reduced size, a lawn of denticles and missing head structures (Fig. 1h, i). Heterozygosity for the entire fourth chromosome or for the chromosomal deletion *Df(4)M62f* suppresses *arm* mutations whereas heterozygosity for *Df(4)M63a* does not. Two genes in the suppressing region, *ci* and *dTcf*, are required for normal segment polarity; *ci* acts in Hedgehog signalling[16,17]. Two *dTcf* null alleles strongly suppress the arm^{XP33} mutation (Fig. 1j, k), as does ci^{D}, a *ci dTcf* double mutant, whereas ci^{Cell}, a putative *ci* null, does not. Suppression is not allele-specific; *dTcf* suppresses the zygotic null arm^{YD35} (Fig. 1l, m) and the moderate hypomorph arm^{XM19} (Table 1).

We previously constructed a dTcf molecule (dTcf-ΔN), lacking the putative Arm-binding domain, that antagonizes Wg signalling when ubiquitously expressed during embryogenesis[8]. The antagonism depends on expression level, ranging from a weak phenotype,

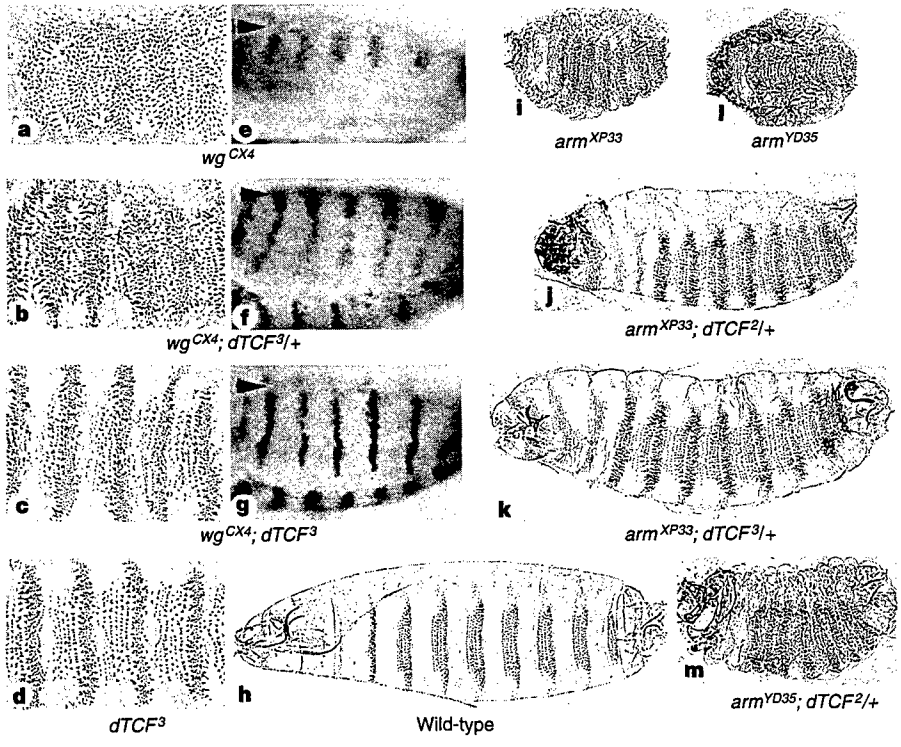


Figure 1 *dTcf* is a dose-dependent suppressor of *wg* and *arm*. In all photographs, anterior is to the left. **a**, wg^{CX4} null homozygote. **b**, wg^{CX4};$dTcf^{3}$/+ embryos show partial suppression of the *wg* phenotype ($n > 100$). **c**, wg^{CX4};$dTcf^{3}$ embryos show more extensive suppression ($n > 300$). **d**, $dTcf^{3}$ homozygotes resemble wg^{CX4};$dTcf^{3}$. **e**, wg^{CX4} mutant embryos lose epidermal En antibody staining (En staining in nervous system is below the plane of focus). The arrowhead indicates the posterior ventral midline. **f**, wg^{CX4};$dTcf^{3}$/+ embryos retain some En-staining cells ($n > 100$). **g**, En is further derepressed in wg^{CX4};$dTcf^{3}$ double homozygotes ($n > 200$). **h**, Wild-type embryo (**h**–**m** are at the same magnification, lower than that in **a**–**g**). **i**, arm^{XP33}/Y. **j**, arm^{XP33}/Y;$dTcf^{2}$/+, and **k**, arm^{XP33}/Y;$dTcf^{3}$/+ show partial suppression of the arm^{XP33}/Y phenotype. **l**, arm^{YD35}/Y. **m**, arm^{YD35}/Y; $dTcf^{2}$/+ shows substantial suppression of the arm^{YD35}/Y phenotype.

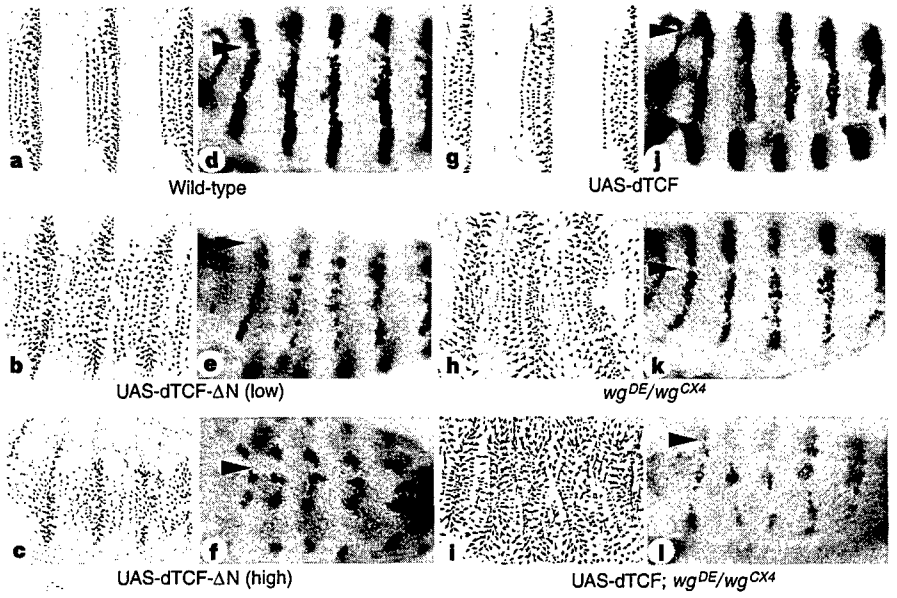


Figure 2 dTcf represses Wg-responsive genes. **a**–**f**, dTcf-ΔN is a constitutive repressor. **a**, A UAS-dTcf-ΔN construct without a Gal4 driver produces a wild-type phenotype. UAS-dTcf-ΔN expressed ubiquitously with E22C-GAL4 produces **b**, moderate to **c**, severe segment polarity phenotypes. **d**, Normal En antibody staining in a stage 10 embryo containing UAS-dTcf-ΔN without a Gal4 driver. The arrowhead indicates the posterior ventral midline. UAS-dTcf-ΔN expressed ubiquitously reduces (**e**) or eliminates (**f**) epidermal *en* expression. **g**–**l**, Wild-type dTcf acts as a repressor when Wg signalling is limited. **g**, Ubiquitous UAS-dTcf changes the wild-type cuticle pattern only subtly. **h**, *Df(2)DE/wg*^{CX4} embryos show a weak *wg* phenotype. **i**, Ubiquitous UAS-dTcf expression in *Df(2)DE/wg*^{CX4} embryos results in a severe *wg* phenotype. **j**, Ubiquitous UAS-dTcf does not disrupt wild-type *en* expression. **k**, *Df(2)DE/wg*^{CX4} embryos show slightly reduced En staining. **l**, En staining in *Df(2)DE/wg*^{CX4} embryos is repressed by ubiquitous UAS-dTcf.

similar to that produced by the *dTcf* zygotic null allele (Fig. 2a, b), to a strong phenotype resembling that resulting from a *wg* null allele (Fig. 2c). Ubiquitous dTcf-ΔN expression also reduces *en* expression; the normal epidermal En stripe (Fig. 2d) is reduced to scattered *en*-expressing cells (Fig. 2e) or is completely repressed (Fig. 2f), mimicking *wg* loss of function. Full-length dTcf does not have this effect (Fig. 2g). Thus, dTcf-ΔN, lacking the Arm-binding region, acts as a constitutive repressor. This reconciles our results with those of ref. 6, in which *dTcf* mutations were isolated as suppressors of *wg* hyperactivity. These *dTcf* alleles contained amino-terminal missense mutations which reduced Arm-binding[6], and, thus, should selectively disrupt dTcf's activation function but leave intact its repressive function.

Thus the difference between dTcf in its role as activator versus repressor seems to reflect a balance between dTcf with and without Arm. Overexpression of full-length dTcf in a normal embryo does not antagonize Wg signalling (Fig. 2g, j). However, when levels of Arm are reduced by limiting Wg activity, similar overexpression of full-length dTcf represses Wg target genes. We lowered Wg activity by using *Df(2)DE*[18], which removes part of the *wg* regulatory region (A.B., data not shown); Wg signalling is reduced but still specifies many wild-type pattern elements (Fig. 2h) and stabilizes some epidermal *en* expression (Fig. 2k). Overexpression of full-length dTcf in these embryos both eliminates wild-type pattern elements (Fig. 2j) and represses *en* expression (Fig. 2l). As overexpression of dTcf has no effect on *en* expression in wild-type embryos (Fig. 2j), we conclude that whether dTcf acts as an activator or a repressor depends on the level of Wg signalling and probably on the amount of available Arm.

In cultured mammalian cells, dTcf alone does not repress transcription of reporter genes[8]. Thus, it seemed likely that dTcf requires a corepressor, as it requires Arm as a coactivator. We have found that Tcf-1 binds to vertebrate Grg family members, homologues of *Drosophila* Groucho (Gro), a known corepressor[19]. We therefore tested whether *Drosophila* Gro binds dTcf (Fig. 3). When we expressed the N-terminal region of *Drosophila* Gro (amino acids 1–181) in COS cells, it localized to the cytoplasm (Fig. 3a).

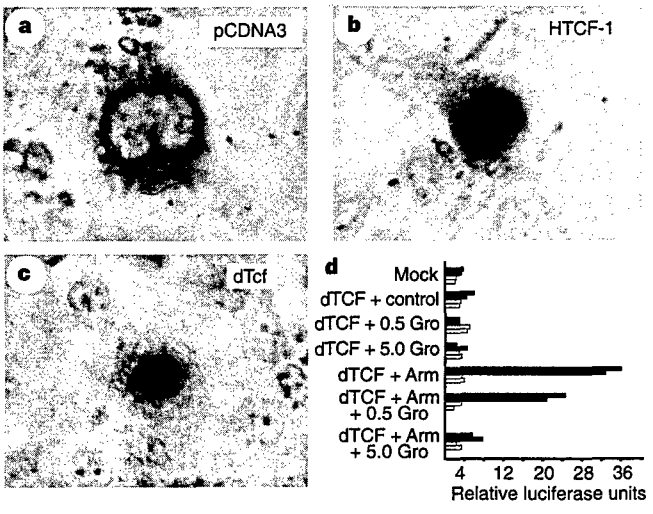


Figure 3 dTcf interacts with *Drosophila* Gro. **a**, A Myc-tagged N-terminal fragment of Gro protein (amino acids 1–181) localizes to the cytoplasm of COS cells. Co-transfection with **b**, human Tcf-1 or **c**, *Drosophila* Tcf results in nuclear localization of Gro(1–181). **d**, Arm-dTcf-mediated transactivation of a dTcf reporter gene is repressed by Gro. IIAI.6 B cells were transfected with the indicated expression plasmids. Shaded bars indicate luciferase activity of pTKTOP, a reporter plasmid containing three optimal dTcf-binding sites upstream of the minimal HSV-TK promoter; unshaded bars indicate activity of pTKFOP, a similar construct with mutated dTcf-binding sites.

Coexpression of either human Tcf-1 (Fig. 3b) or dTcf (Fig. 3c) results in the localization of Gro(1–181) to the nucleus, consistent with a physical association between the proteins. Full-length Gro is constitutively nuclear (data not shown), and, as such, is not informative in this assay. The nuclear recruitment of Gro(1–181) by dTcf is very similar to the recruitment of β-catenin[20], a known Tcf-binding partner. We then determined whether the dTcf–Gro

association has functional consequences (Fig. 3d). We transfected IIAI.6 B cells with reporter genes containing dTcf-binding sites upstream of the thymidine kinase (TK) promoter. Expression of dTcf alone leads to very little reporter activation, whereas coexpression of dTcf and Arm leads to robust activation[8]. Expressing *Drosophila* Gro results in a dose-sensitive reversal of coactivation (Fig. 3d), again consistent with a physical interaction.

To determine if Gro acts as a corepressor *in vivo*, we tested whether *gro* mutants interact genetically with the Wg signalling pathway. *Drosophila* Gro acts as a corepressor for several transcription factors and is essential for dosage compensation, early segmentation and neurogenesis[21]. These pleiotropic effects would obscure later effects on epidermal patterning by the Wg pathway. However, as with *dTcf*, we find that *gro* mutations show dose-sensitive interactions with both *wg* and *arm*. Reducing the dose of maternal Gro suppresses the *wg* null phenotype, whereas reduction of paternal Gro has no effect (Fig. 4a, c). Zygotic reduction does not substantially increase rescue of the *wg* null phenotype (Fig. 4e). Suppression of this phenotype correlates with partial derepression of epidermal *en* expression (Fig. 4b, d). Reduction of both maternal and zygotic Gro also strongly suppresses *arm* phenotypes (Fig. 4k–o), whereas reduction of zygotic Gro alone does not. Two other aspects of the *gro* phenotype support a function for Gro in Wg signalling. First, some zygotically mutant *gro* embryos retain sufficient ventral epidermis to secrete a cuticle pattern exhibiting subtle patterning defects, consistent with mild Wg hyperactivation (Fig. 4f). Second, *gro* zygotic mutants show expanded *en* expression[22], as seen when Wg is hyperactivated[23].

If dTcf and Gro act together to mediate repression, dTcf repression should require Gro. We thus tested whether a reduction in Gro dosage diminishes dTcf's effectiveness as a repressor. Excess dTcf represses Wg target genes in weak *wg* mutant embryos (Fig. 2i, k, l). However, when maternal Gro levels are reduced, dTcf repression is decreased significantly (Fig. 4g, h). Gro also mediates the repression produced by dTcf-ΔN, which lacks the Arm-binding region but retains the putative Gro-binding site[19]. Ectopic expression of dTcf-ΔN in wild-type embryos antagonizes Wg signalling. Reduction in maternal Gro levels significantly reduces the ability of dTcf-ΔN to act as a repressor (Fig. 4i, j and Table 1).

Our data indicate that dTcf has a negative as well as a positive

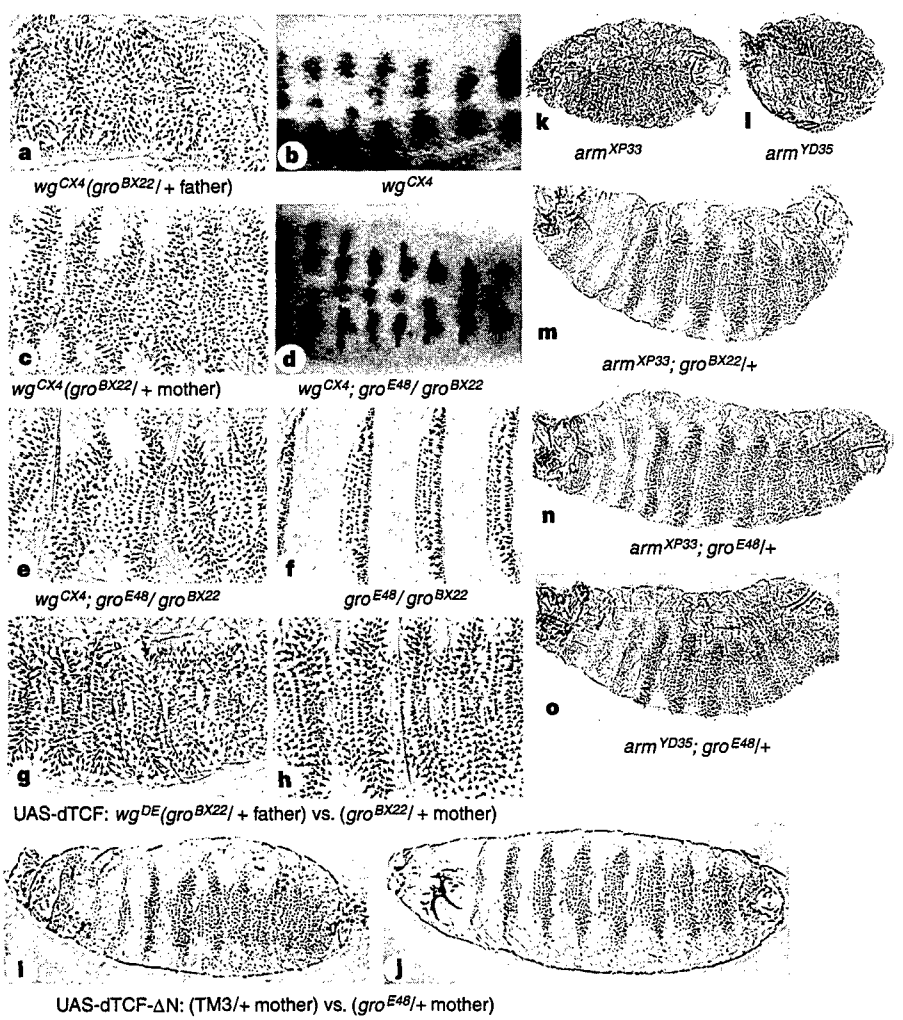


Figure 4 Gro acts together with dTcf to repress Wg-responsive genes. **a**, **b**, A reduction in levels of zygotic Gro alone has no effect: wg^{CX4};gro^{BX22}/+ embryos from gro^{BX22}/+ fathers are indistinguishable from *wg* null mutants in cuticle pattern (**a**, $n > 100$) and *en* expression (**b**, ventral view, stage 13 *wg* mutant). **c**, wg^{CX4};gro^{BX22}/+ embryos from gro^{BX22}/+ mothers show suppression (203 of 207 embryos). **d**, both wg^{CX4};gro^{BX22}/+ embryos from gro^{BX22}/+ mothers and wg^{CX4};gro^{E48}/gro^{BX22} embryos (shown) maintain ventral epidermal *en* expression. **e**, wg^{CX4};gro^{E48}/gro^{BX22} shows similar rescue of pattern elements compared with *wg* mutants. **f**, gro^{E48}/gro^{BX22} embryos show subtle pattern alterations. **g**, **h**, dTcf overexpression in $Df(2)DE/wg^{CX4}$ embryos produces a strong *wg*-like phenotype, even when embryos are zygotically *gro*/+ (**g**, $n > 100$), but not when embryos are maternally *gro*/+ (**h**, 209 of 223 embryos resemble $Df(2)DE/wg^{CX4}$ embryos). **i**, **j**, Ubiquitous dTcf-ΔN produces a moderate segment polarity phenotype (**i**), which is suppressed in embryos with *gro*/+ mothers (**j**). **k**–**o**, Heterozygosity of *gro* substantially suppresses *arm* mutations. **k**, arm^{XP33}/Y and **l**, arm^{YD35}/Y embryos show a strong segment polarity phenotype. **m**, arm^{XP33}/Y;gro^{BX22}/+, **n**, arm^{XP33}/Y; gro^{E48}/+, and **o**, arm^{YD35}/Y; gro^{E48}/+, all from *arm* and *gro* heterozygous mothers, show suppression of the strong segment polarity phenotype.

function in transcriptional regulation, and that Gro acts in a repressor complex with dTcf. This dual regulatory role may be conserved in vertebrate Wnt signalling[9,19]. Therefore, we propose that the balance between the activity of Gro and Arm controls cell-fate choice by the Wnt pathway in both vertebrates and invertebrates. □

Methods

Fly stocks and crosses. Cuticle preparations and antibody stainings were performed as described[24]. In Figs 1, 2 and 4, genotypes were assigned by comparing the frequencies of phenotypic classes with expected genotypic frequencies; these data are summarized in Table 1. For *arm*, suppression was documented by ranking embryos in weak to strong phenotypic categories and calculating ratio of embryos in weak categories. wg^{CX4} is a molecular null allele[25]; *Df(2)DE*[18] is a *wg* hypomorph (A.B., unpublished observations); arm^{XP33} is a strong hypomorph; arm^{YD35} is a null allele[15]; both *dTcf* mutations used are molecular null alleles[8]; gro^{E48} is a putative null point mutation[21]; gro^{BX22} lacks *gro* and several neighbouring genes in the *Enhancer of split* complex[26]. Gal4 and UAS transgene stocks have been described[8].

Mammalian cell culture. Vector alone (pCDNA3), hTcf-1 or dTcf and Myc-epitope-tagged Gro(1–181) constructs (with a ratio of 10:1) were introduced into COS cells by diethyl aminoethyl-dextran transfections. Cells were prepared for immunohistochemistry using an anti-Myc-antibody. 2×10^6 IIAI.6 B cells were transfected by electroporation with 1 μg dTcf luciferase reporter plasmid (pTKTOP) or its negative control containing mutated dTcf sites (pTKFOP)[19]. These were co-transfected with 2 μg dTcf expression vector, 0.5 or 5.0 μg Gro expression plasmids and 0.5 μg Arm expression plasmid, balanced to equal plasmid amounts with pCDNA3. Luciferase activity was corrected by chloramphenicol acetyltransferase (CAT) activity[19]. Luciferase and CAT activities were determined as in ref. 8.

Received 15 June; accepted 5 August 1998.

1. Cadigan, K. M. & Nusse, R. Wnt signaling: a common theme in animal development. *Genes Dev.* **11**, 3286–3305 (1997).
2. Bejsovec, A. & Peifer, M. The *wingless/Wnt-1* signaling pathway—new insights into the cellular mechanisms of signal transduction. *Adv. Dev. Biochem.* **4**, 1–45 (1996).
3. Rubinfeld, B. *et al.* Stabilization of β-catenin by genetic defects in melanoma cell lines. *Science* **275**, 1790–1792 (1997).
4. Morin, P. J. *et al.* Activation of β-catenin-Tcf signalling in colon cancer by mutations in β-catenin or APC. *Science* **275**, 1787–1790 (1997).
5. Korinek, V. *et al.* Constitutive transcriptional activation by a β-catenin-Tcf complex in APC(–/–) colon carcinoma. *Science* **275**, 1784–1787 (1997).
6. Brunner, E., Peter, O., Schweizer, L. & Basler, K. *pangolin* encodes a Lef-1 homolog that acts downstream of Armadillo to transduce the Wingless signal. *Nature* **385**, 829–833 (1997).
7. Riese, J. *et al.* LEF-1, a nuclear factor coordinating signalling inputs from *wingless* and *decapentaplegic*. *Cell* **88**, 777–787 (1997).
8. van de Wetering, M. *et al.* Armadillo co-activates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. *Cell* **88**, 789–799 (1997).
9. Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T. & Kimelman, D. A. β-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Genes Dev.* **11**, 2359–2370 (1997).
10. DiNardo, S., Kuner, J. M., Theis, J. & O'Farrell, P. H. Development of the embryonic pattern in *D. melanogaster* as revealed by the accumulation of the nuclear *engrailed* protein. *Cell* **43**, 59–69 (1985).
11. DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J. A. & O'Farrell, P. Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* **322**, 604–609 (1988).
12. Martinez Arias, A., Baker, N. & Ingham, P. Role of the segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**, 157–170 (1988).
13. Bejsovec, A. & Martinez-Arias, A. Roles of *wingless* in patterning the larval epidermis of *Drosophila*. *Development* **113**, 471–485 (1991).
14. Heemskerk, J., DiNardo, S., Kostriken, R. & O'Farrell, P. H. Multiple modes of *engrailed* regulation in the progression towards cell fate determination. *Nature* **352**, 404–410 (1991).
15. Peifer, M. & Wieschaus, E. The segment polarity gene *armadillo* encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. *Cell* **63**, 1167–1178 (1990).
16. Von Ohlen, T., Lessing, D., Nusse, R. & Hooper, J. E. Hedgehog signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein. *Proc. Natl Acad. Sci. USA* **94**, 2404–2409 (1997).
17. Hepker, J., Wang, Q. T., Motzny, C. K., Holmgren, R. & Orenic, T. V. *Drosophila* cubitus interruptus forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. *Development* **124**, 549–558 (1997).
18. Tiong, S. Y. K. & Nash, D. Genetic analysis of the *adenosine3* (*Gart*) region of the second chromosome in *Drosophila melanogaster*. *Genetics* **124**, 889–897 (1990).
19. Roose, J. *et al.* The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**, 608–612 (1998).
20. Behrens, J. *et al.* Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* **382**, 638–642 (1996).
21. Paroush, Z. *et al. groucho* is required for *Drosophila* neurogenesis, segmentation, and sex determination and interacts directly with Hairy-related bHLH proteins. *Cell* **79**, 805–815 (1994).
22. de Celis, J. F. & Ruiz-Gomez, M. *groucho* and *hedgehog* regulate *engrailed* expression in the anterior compartment of the *Drosophila* wing. *Development* **121**, 3467–3476 (1995).
23. Noordermeer, J., Johnston, P., Rijsewijk, F., Nusse, R. & Lawrence, P. A. The consequences of ubiquitous expression of the *wingless* gene in the *Drosophila* embryo. *Development* **116**, 711–719 (1992).
24. Bejsovec, A. & Wieschaus, E. Signaling activities of the *Drosophila wingless* gene are separately mutable and appear to be transduced at the cell surface. *Genetics* **139**, 309–320 (1995).
25. Baker, N. E. Molecular cloning of sequences from *wingless*, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. *EMBO J.* **6**, 1765–1773 (1987).
26. Schrons, H., Knust, E. & Campos-Ortega, J. A. The *Enhancer of split* complex and adjacent genes in the 96F region of *Drosophila melanogaster* are required for segregation of neural and epidermal progenitor cells. *Genetics* **132**, 481–503 (1992).

Acknowledgements. This work was supported by grants from the NIH and the U.S. Army Breast Cancer Research Program (to M.P.), the NSF and the March of Dimes Birth Defects Foundation (to A.B.) and the NWO-GMW (to H.C.).

Correspondence and requests for materials should be addressed to A.B. (e-mail: bejsovec@nwu.edu).